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Coordination of Reproductive Activity in *Aplysia:* Peptide Neurohormones, Neurotransmitters, and Pheromones Encoded by the Egg-Laying Hormone Family of Genes

SHERRY D. PAINTER

The Marine Biomedical Institute and The Department of Anatomy and Neurosciences, University of Texas Medical Branch, Galveston, Texas 77555-0843

Abstract. Pheromones play a significant role in coordinating reproductive activity in the marine opisthobranch mollusk *Aplysia.* Although solitary during most of the year, these simultaneous hermaphrodites gather into breeding aggregations during the reproductive season. The aggregations contain both mating and egg-laying animals, and are associated with masses of recently deposited egg cordons. Behavioral studies suggest that cordon-derived pheromonal factors are primarily responsible for establishing and maintaining the aggregations. Egg-laying animals are more attractive than sexually mature, but nonlaying, conspecifics and have a shorter mean latency to mating; egg cordons and egg-cordon eluates, when placed in the surrounding seawater, enhance the attractiveness of nonlaying animals and reduce their mean latency to mating. Similar effects are observed when extracts of the atrial gland are placed in the seawater, suggesting that secretory products of this oviductal exocrine organ may function as sexual pheromones. Biochemical analyses indicate that there may be multiple attractants in atrial gland extracts, and that at least one of these (A-NTP) is a peptide encoded by the A gene. The A gene belongs to a small family of structurally related genes that are expressed in a tissue-specific manner. Another member of the family, the egg-laying hormone (ELH) gene, is expressed in the neuroendocrine bag cells. Peptide products of the ELH gene act as neurohormones and nonsynaptic neurotransmitters, initiating egg laying and coordinating its associated behaviors. Peptide products of a family of genes may thus act internally and externally to coordinate both male and female reproductive activities.

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Abbreviations: AG, atrial gland; ASW, artificial seawater; BCP, bag-cell peptide; ELH, egg-laying hormone; NTP, N-terminal peptide; RHD, red hemiduct; WHD, white hemiduct.

Introduction

The neuroendocrine regulation of reproductive activity in the marine opisthobranch mollusk *Aplysia* has been widely studied for over 25 years. Attention has focused primarily on the induction of egg laying and the regulation of its associated behaviors. Although pheromones play a significant role in coordinating reproductive activity in this genus, relatively little is known about the pheromonal factors or their specific activities.

Field studies of both *A. californica* (Kupfermann and Carew, 1974; Audesirk, 1979) and *A. fasciata* (Susswein *et al.*, 1983, 1984) indicate that, although *Aplysia* is a solitary animal during most of the year, it gathers into breeding aggregations or "brothels" during the summer reproductive season. The aggregations, containing both mating and egg-laying animals, are usually associated with masses of recently deposited egg cordons, often laid one on top of another. Most of the egg-laying animals simultaneously mate as females, even though mating does not cause reflex ovulation (*A. brasiliana,* Blankenship *et al.*, 1983), suggesting that egg laying may precede mating in the aggregations rather than resulting from it. Similar observations have been made in the laboratory when animals have not been individually caged (*A. californica,* Audesirk, 1977; *A. fasciata,* Susswein *et al.*, 1983, 1984; *A. brasiliana,* Blankenship *et al.*, 1983).

Behavioral studies suggest that the egg cordon is a source of pheromonal factors that attract conspecifics and induce

mating activity (*A. brasiliana*, Aspey and Blankenship, 1976; Jahan-Parwar, 1976; Painter *et al.*, 1989, 1991; *A. californica*, Jahan-Parwar, 1976; Audesirk, 1977); they may also induce egg-laying activity (*A. californica*, Audesirk, 1977) and may be responsible for the masses of egg cordons associated with breeding aggregations. Because mating does not enhance the attractiveness of a nonlaying animal (*A. dactylomela*, Lederhendler *et al.*, 1977), and mating does not cause reflex ovulation (*A. brasiliana*, Blankenship *et al.*, 1983), egg cordons are likely to be the primary sources of pheromonal factors that establish and maintain the aggregations.

Behavioral studies further suggest that the atrial gland, an exocrine organ secreting into the oviduct (*A. californica*, Arch *et al.*, 1980; Beard *et al.*, 1982; Painter *et al.*, 1985), may be a tissue source of the "cordon-derived" pheromonal activity (Painter *et al.*, 1989, 1991; also see Susswein and Benny, 1985). Extracts of other regions of the reproductive tract have not been examined, however, and the active atrial gland factors have not been identified.

The experiments described in this report demonstrate that extracts of the atrial gland specifically enhance the attractiveness of nonlaying animals, that the activity is found predominantly in the low and intermediate molecular weight fractions examined, and that A-NTP (N-terminal peptide encoded by the A gene) is one of the active factors. The A gene belongs to a small family of structurally related genes that are expressed in a tissue-specific manner in the animal (*A. californica*, Scheller *et al.*, 1983; Mahon *et al.*, 1985). Another member of the family, the egg-laying hormone (ELH) gene, is expressed in the neuroendocrine bag cells; products of the ELH gene appear to act as neurohormones and nonsynaptic neurotransmitters to induce ovulation (*A. californica*, Chiu *et al.*, 1979; Rothman *et al.*, 1983b; *A. brasiliana*, Nagle *et al.*, 1988a), regulate packaging and transport of the eggs through the reproductive tract (*A. californica*, Nagle *et al.*, 1990; Alevizos *et al.*, 1991), and (presumably) coordinate a stereotypical series of behaviors that accompany egg deposition. Thus, peptide products of a family of genes may act both internally and externally to coordinate male and female reproductive activities, ensuring propagation of the species.

Materials, Methods, and Results

Animals

Specimens of *Aplysia brasiliana* (Rang), weighing from 95 to 400 g, were collected from South Padre Island, Texas, and were used in experiments between June and September, the normal reproductive season for this species. *Aplysia brasiliana* was selected as the experimental animal for T-maze experiments because it has lower levels of chance attraction than *A. californica* (*i.e.*, it is less likely to enter one of the arms and stop locomoting when no attractant is present; Painter, 1991), and it can be collected in large numbers from the south Texas coast during the reproductive season. The animals were housed in individual cages in one of five large aquaria containing recirculating artificial seawater (ASW; Instant Ocean, Aquarium Systems, Mentor, Ohio) at room temperature (20 ± 2°C); the salinity ranged from 30 to 32 ppt. A 14:10 light:dark cycle was maintained, with the light period starting at 6 am; animals were fed dried laver in the late afternoon (4–6 pm) after the experiments were completed.

Specimens of *Aplysia californica* (Cooper) were purchased from Alacrity Marine Biological Services (Redondo Beach, California) and were maintained as described above for *A. brasiliana*, except that the ASW was cooled to 14 ± 2°C. *Aplysia californica* was used as the source of tissues for biochemical analyses because the exocrine organs associated with the egg-laying portion of the reproductive tract are larger and more well-defined than in *A. brasiliana* (Painter *et al.*, 1985). Note that pheromonal attractants do not appear to be species-specific in *Aplysia* (Kupfermann and Carew, 1974).

Only sexually mature individuals were used as experimental animals or as sources of tissues for biochemical studies. Sexual maturity was defined as the ability to lay eggs spontaneously or in response to injections of atrial gland extract. The extract was prepared as follows. Atrial glands were removed from sexually mature specimens of *Aplysia californica* (approximately 100 mg wet weight per animal) and stored at −70°C until use. Ten glands were homogenized in 20 ml of ice-cold filtered ASW in a hand-held glass-on-glass homogenizer. The homogenate was centrifuged at 48,000 × *g* for 20 min at 4°C, the supernatant collected and frozen at −20°C until use. Egg deposition was induced by injecting 0.1 ml of the thawed extract through the foot into the hemocoel; egg laying began approximately 30 min later. Most animals were injected two to five days after arriving in the lab. Immature animals were injected at weekly intervals thereafter until they responded to the injections.

Bioassay

Apparatus. As previously described (Painter *et al.*, 1991), a T-maze was constructed of clear Plexiglas (0.62 cm thick) and sealed with clear aquarium cement; it was cured for several days in ASW before use. Its overall dimensions were: height (base to top of T), 40.7 cm; width (distance between ends of arms), 101 cm; width of pathway, 10.2 cm; and depth, 10.2 cm. Removable stimulus cages (12.7 cm × 10 cm; depth, 10.2 cm) were placed in both arms for each experiment.

Experimental protocol and statistical analyses. Experiments were performed in a room adjacent to the aquar-

ium facility with overhead fluorescent lighting; the maze was positioned so that the lighting was uniform throughout the apparatus. As previously described (Painter *et al.*, 1991), 6 l of aerated ASW that had not previously contacted an *Aplysia* was placed in a cleaned and air-dried maze. A sexually mature "stimulus" animal was placed in a stimulus cage in one arm and a potential attractant added to the adjacent seawater. A sexually mature "test" animal was placed in the base of the maze 5 min later. Both the stimulus and test animals were briefly rinsed in fresh ASW before being introduced into the maze. In most cases, the test animal moved directly to the top of the maze and exhibited one of two general types of behavior: (1) it stopped, moved its head from side to side, then moved into one arm or returned to the base of the maze and stayed; or (2) it swam back and forth between arms, sometimes returning to the base, until it decided to stop. A response was considered to be positive if the test animal travelled to the stimulus within 20 min and maintained contact with the stimulus cage for 5 min, negative if it travelled to the opposite arm and maintained contact with the cage for 5 min, and no choice if it did neither. Animals were choosing between a stimulus and no stimulus in these experiments, rather than between two qualitatively or quantitatively different stimuli. Fifteen experiments were performed for each potential attractant, and the attractant was alternated between arms in consecutive experiments. Statistical significance was assessed by χ^2 analyses.

Four criteria were used to select animals for each experiment: (1) the animal must not have laid eggs during the preceding 24 h; (2) the animal must not have participated in a behavioral experiment during the preceding 24 h; (3) the animal must not have served as a test animal for the attractant being examined; and (4) the test and stimulus animals must have been housed in the same aquarium.

Tissue sources of pheromonal attractants

Tissue extracts for bioassay. Reproductive tracts were removed from sexually mature animals that had not laid eggs during the preceding 24 h. They were dissected into 6 components: the albumen gland, mucous gland, winding gland, atrial gland, red hemiduct (RHD), and white hemiduct (WHD) (Fig. 1). The albumen, mucous and winding glands are components of the accessory genital mass, and are responsible for packaging the eggs into a cordon (*A. californica*, Coggeshall, 1972). The RHD and atrial gland are components of the functional oviduct, through which the egg cordon is transported to the exterior of the animal. The WHD is the copulatory portion of the tract and does not contribute directly to egg deposition (*A. californica*, Painter *et al.*, 1985). Tissues were stored at −70°C until use.

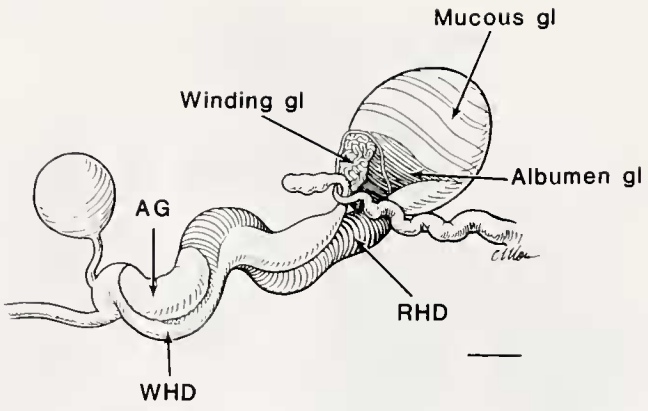

Figure 1. Schematic diagram of the reproductive tract of *Aplysia californica* anterior to the ovotestis. Extracts were made of each of the labeled organs, and the extracts bioassayed for pheromonal activity in T-maze experiments. RHD: red hemiduct; WHD: white hemiduct.

Prior to extraction, the tissues were thawed and weighed. They were then homogenized in ice-cold filtered ASW (3 cycles, final volume: 1 ml/50 mg wet weight) in a hand-held glass-on-glass homogenizer, and the homogenate centrifuged at 48,000 × *g* for 20 min at 4°C. The supernatant was collected, distributed into 1-ml aliquots, and the aliquots frozen at −20°C until used in a bioassay (3–6 days for each tissue).

Animals. Ninety-five *Aplysia brasiliana* were used in these experiments.

Results. To assess directional bias and chance levels of attraction in the maze, 15 experiments were performed in which no stimulus was placed in either arm. Four animals (26.7%) moved to the left and remained, two (13.3%) moved to the right and remained, while 9 (60%) did neither. These results demonstrate that there is no directional bias in the maze and establish chance levels of attraction at three animals (20%).

A similar level of attraction (2 animals; 13.3%) and pattern of responses was obtained when the stimulus was a nonlaying animal (Fig. 2). The two sets of responses were not significantly different [$\chi^2(2) = 0.92$, $0.50 < P < 0.75$], consistent with results obtained in earlier studies using this bioassay system (Painter *et al.*, 1991).

In subsequent experiments, aimed at enhancing the attractiveness of a nonlaying stimulus animal, extracts of various reproductive tract organs were placed in the adjacent ASW. Extracts of either the albumen gland or the atrial gland increased the number of animals attracted to the nonlayer and decreased the number of animals making no choice responses (Fig. 2). The pattern of responses obtained with the atrial gland extract differed significantly from that obtained with the nonlayer alone ($\chi^2 = 8.91$, $0.01 < P < 0.025$); the changes in pattern obtained with the albumen gland extract were less significant [$\chi^2(2) = 3.98$, $P = 0.20$].

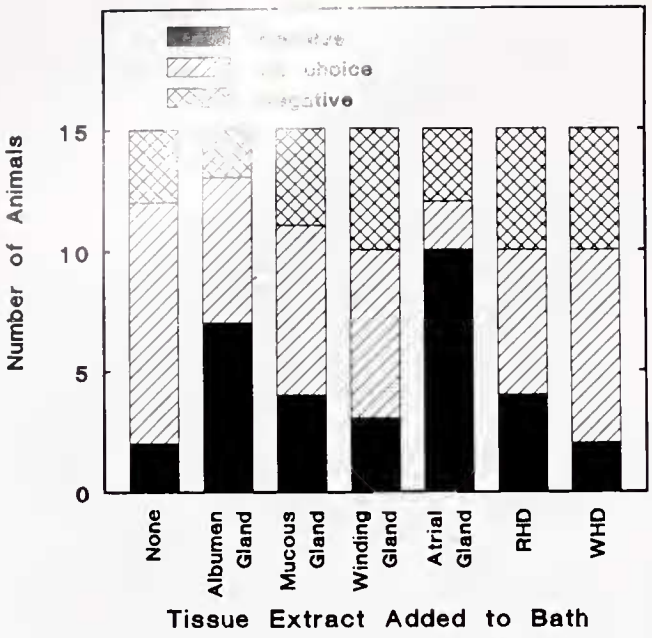

Figure 2. Secretory products of both the *Aplysia californica* albumen gland and atrial gland may be pheromonal attractants. The number of *A. brasiliana* attracted to a nonlaying conspecific was increased when an extract of either the albumen gland or atrial gland was placed in the adjacent ASW; only the atrial gland significantly altered the pattern of responses. Extracts of other *A. californica* reproductive organs did not affect the attractiveness of a nonlaying animal or significantly affect the pattern of responses. This bar graph is based on 105 single-arm experiments, 15 per stimulus; in each experiment, animals chose between a stimulus in one arm and no stimulus in the other.

Extracts of other reproductive tract organs did not affect the number of animals attracted to the nonlayer or the number of animals making no choice responses (Fig. 2). In each case, the pattern of responses did not differ significantly from that obtained with the nonlayer alone [$\chi^2(2) = 1.08$, $0.50 < P < 0.75$ for the mucous gland; $\chi^2(2) = 0.46$, $0.75 < P < 0.90$ for the winding gland; $\chi^2(2) = 2.16$; $0.25 < P < 0.50$ for the RHD; $\chi^2(2) = 0.72$, $0.50 < P < 0.75$ for the WHD]. These results suggest that the enhanced attractiveness of the nonlaying animal was specifically associated with the atrial gland extracts, and that it may be a physiological source of "cordon-derived" pheromonal attractants.

Extracts and column chromatography. In this case, six atrial glands (800 mg wet weight) were homogenized in 10 ml of 1 *M* acetic acid containing 20 m*M* HCl (4°C) in a hand-held glass-on-glass homogenizer. The homogenate was centrifuged at 48,000 × *g* for 20 min at 4°C, the supernatant removed and immediately applied to a 2.5 cm × 49 cm column of Sephadex G-50 superfine (4°C). The column had been calibrated with the following molecular weight standards: bovine serum albumin (66,200), cytochrome *c* (12,300), bacitracin (1500), and cobalt chloride (660). The eluting material was pooled into three fractions (I, >10 kDa; II, 1.5–10 kDa; III, <1.5 kDa); each fraction was then distributed into 16 aliquots and lyophilized. The lyophilized aliquots were resuspended in distilled water, relyophilized, and frozen at −20°C until use. Each was resuspended in 1 ml of distilled water immediately before addition to the maze.

Results. Sephadex G-50 elution profiles of acidic extracts of the atrial gland have been published previously (see, *e.g.,* Nagle *et al.,* 1988b) and are not shown here. Each of the molecular weight fractions increased the number of animals attracted to the nonlayer and decreased the number of animals making no choice responses (Fig. 3), but only fraction II significantly altered the pattern of responses from that obtained with the nonlayer alone [I, $\chi^2(2) = 1.76$, $0.25 < P < 0.50$; II, $\chi^2(2) = 9.34$, $P < 0.01$; III, $\chi^2(2) = 5.60$, $P = 0.06$]. The pattern of responses to fraction III did not differ significantly from that to fraction II [$\chi^2(2) = 1.45$, $0.25 < P < 0.50$] and none of the patterns differed significantly from the pattern obtained with a nonlayer and unfractionated atrial gland extract [I, $\chi^2(2) = 3.58$; $0.10 < P < 0.25$; II, $\chi^2(2) = 1.15$; $0.50 < P < 0.75$; III, $\chi^2(2) = 3.06$, $0.10 < P < 0.25$]. These results suggest that the atrial gland attractants have a wide range of molecular weights.

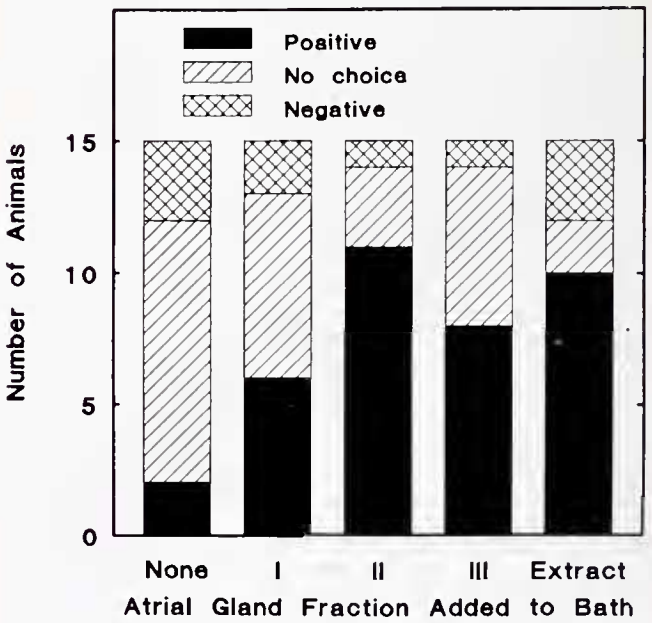

Figure 3. Atrial gland attractant activity is distributed over a range of molecular weights: the number of *A. brasiliana* attracted to a nonlaying conspecific was increased by placing fraction I (>10 kDa), II (1.5–10 kDa) or III (<1.5 kDa) in the adjacent ASW. The patterns of responses were not significantly different from that to a nonlayer with unfractionated atrial gland extract. This bar graph is based on 75 single-arm experiments, 15 per stimulus; in each experiment, animals chose between a stimulus in one arm and no stimulus in the other.

Identification of A-NTP as a potential attractant

Previous studies (Nagle *et al.*, 1988b) have shown that the low molecular weight fraction III contains two predominant peptide species, corresponding to A-NTP (N-terminal peptide encoded by the A gene) and B-NTP (N-terminal peptide encoded by the B gene). These peptides are each 13 residues in length, differing in sequence at only 3 positions, and have blocked amino termini (Fig. 4). Because amino acid compositional and microsequence analyses indicate that A-NTP is present in significantly larger quantities than B-NTP (Nagle *et al.*, 1988b), we decided to synthesize A-NTP for bioassay and future egg-cordon elution experiments.

Peptide synthesis. A-NTP was synthesized at the Biomolecular Synthesis Facility at the University of Texas Medical Branch and purified by C18 reversed-phase HPLC. The identity of the peptide was confirmed, and the amount of material quantified by amino acid compositional analysis. Microsequence analyses (as described in Nagle *et al.*, 1988b) yielded no amino acid residues, confirming that the amino terminus was blocked.

Bioassay. Three stimuli were examined in this series of experiments: (1) a nonlayer with nothing added to the ASW; (2) a nonlayer with a recently deposited conspecific egg cordon (laid by another animal following injection of atrial gland extract; deposition completed within the preceding 30 min; mean volume, 2.0 ml); and (3) a nonlayer with 100 μg of synthetic A-NTP (60% of the amount of A-NTP and B-NTP in one gland; Nagle *et al.*, 1988b).

Animals. Animals were selected from a pool of 183.

Results. Because of the larger population of animals used in these experiments, directional bias and chance levels of attraction were reassessed. Four of 15 animals (26.7%) travelled to the left when there was no stimulus in either arm, three of 15 (20%) travelled to the right, and eight (53.3%) did neither. The pattern of responses was not significantly altered when a nonlayer was the stimulus [$\chi^2(2) = 1.70$, $0.25 < P < 0.50$] (Fig. 5).

The number of animals attracted to a nonlayer was increased, and the number of animals making negative or no choice responses decreased, by placing either a conspecific egg cordon or synthetic A-NTP in the adjacent seawater (Fig. 5), but only the egg cordon significantly altered the pattern of responses obtained with nonlaying animals [egg cordon, $\chi^2(2) = 6.92$, $0.025 < P < 0.05$; A-NTP, $\chi^2(2) = 5.64$, $P = 0.06$]. The patterns of responses to these two stimuli did not differ significantly from each other [$\chi^2(2) = 0.09$, $0.95 < P < 0.975$], however. This suggests that pheromonal attractants are highly conserved in the two species of *Aplysia*.

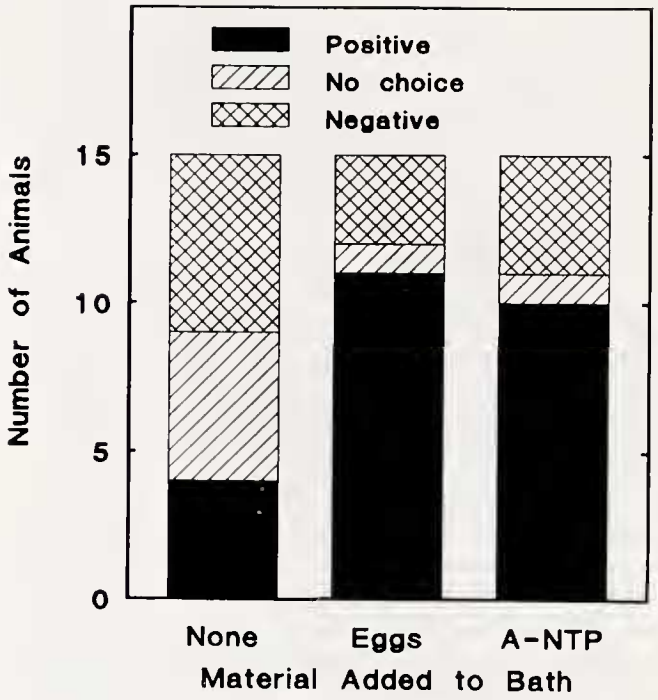

Figure 5. A-NTP is one of the attractive atrial gland factors: the number of *Aplysia brasiliana* individuals attracted to a nonlaying conspecific was increased by placing A-NTP in the adjacent ASW. The response pattern differed significantly from that to a nonlayer alone but not from that to a nonlayer with a recently deposited *A. brasiliana* egg cordon. This bar graph is based on 60 single-arm experiments, 15 per stimulus; in each experiment, animals were choosing between a stimulus in one arm and no stimulus in the other.

A-NTP	pGlu	Ser	Thr	Ser	Val	His	Gly	Lys	Ile	Phe	Val	Pro	Asn
B-NTP	pGlu	Phe	Thr	Ser	Val	Leu	Gly	Lys	Ile	Phe	Val	Thr	Asn

Figure 4. Amino acid sequences of A-NTP and B-NTP, as predicted from nucleotide sequence analyses of the A and B genes (Scheller *et al.*, 1983) and confirmed by biochemical analyses of purified peptides (Nagle *et al.*, 1988b).

Discussion

These experiments show that extracts of the albumen gland and atrial gland enhance the attractiveness of nonlaying animals when placed in the adjacent seawater, but that the pattern of responses is significantly altered only by the atrial gland extracts. This suggests that atrial gland products may play the more significant role in pheromonal attraction. The relative locations of the glands within the reproductive tract would seem to support such a conclusion. The albumen gland is a component of the accessory genital mass and is one of the first exocrine organs contacted by the eggs (*A. californica*, Coggeshall, 1972); the atrial gland, in contrast, is a component of the oviduct and is the last major exocrine organ contacted by the egg cordon before deposition (*A. californica*, Painter *et al.*, 1985). Nevertheless, the material assayed in these exper-

iments was extracted f[illegible]mg wet weight of tissue. This corresponds to a[illegible]ely 40–50% of the material in an atrial gland f[illegible]ally mature animal, but only 10–20% of the [illegible] an albumen gland. Dose-response experi[illegible] not been performed for extracts of either orga[illegible]. It is possible that extracts of 40–50% of the albumen gland would yield more significant changes in response pattern.

Extracts of other portions of the reproductive tract, including the mucous gland, winding gland, RHD, and WHD, did not affect the attractiveness of a nonlaying animal, suggesting that the attractant activity is specifically associated with the atrial gland (and perhaps the albumen gland) rather than being broadly distributed throughout the egg-laying portion of the tract.

The <1.5 kDa and 1.5–10 kDa fractions increased the number of animals attracted to a nonlayer in the maze, demonstrating that attractant activity occurs over a broad range of molecular weights and suggesting that there may be multiple attractants in the gland. One of these is A-NTP, the predominant component of the low molecular weight fraction (Nagle *et al.*, 1988b). The second (and only other) major component of this fraction is B-NTP, a 13-residue peptide that is identical to A-NTP at 10 of the 13 positions (Fig. 4). Although B-NTP has not been tested in the bioassay, it is likely to be attractive as well, and probably contributes to the attractiveness of both the low molecular weight fraction and the unfractionated atrial gland extract.

The amount of A-NTP tested in the bioassay is equivalent to 60% of the material in a single atrial gland and is probably an unphysiologically high dose. This raises the possibility that the attraction is a pharmacological rather than a physiological effect. We have not as yet performed dose-response experiments to determine where this dose falls on the curve, or analyzed egg cordon eluates to determine the amounts of material that might be recovered from an egg cordon. These experiments are in progress, however.

The attractive components of the intermediate molecular weight fraction have not yet been identified. The activity is unlikely to result from complexing of a single low molecular weight species (*e.g.*, A-NTP or B-NTP), since previous studies suggest that complexing is minimized under the acidic conditions used in our experiments (Heller *et al.*, 1980; Kelner *et al.*, 1984). More importantly, complexes have not been identified in biochemical analyses of the 1.5–10 kDa fraction of acidic atrial gland extracts (Nagle *et al.*, 1986, 1988b). The fraction may contain novel factors with attractant activity, but the processing intermediates A-NTP-peptide A and B-NTP-peptide B also elute in this fraction (Nagle *et al.*, 1988b). Experiments are in progress to identify the attractive factors in this fraction.

The small amount of attractant activity associated with the high molecular weight fraction may reside in the A or B prohormones, which elute in this fraction, but this has not been demonstrated. The fraction may also contain novel attractants.

Neuroendocrine regulation of egg laying

Much more is known about the neuroendocrine regulation of egg laying in *Aplysia* than is known about pheromonal attraction or pheromonal induction of mating and egg laying. Ovulation is preceded by a prolonged and synchronous burst of electrical activity in the normally quiescent bag cells (*A. brasiliana,* Dudek *et al.*, 1979), two clusters of neuroendocrine cells located in the abdominal ganglion (*A. californica,* Frazier *et al.*, 1967; *A. brasiliana,* Blankenship and Coggeshall, 1976). The stimulus that normally elicits bag-cell activity is not known, but it appears to originate in the cerebral or pleural ganglia (*A. californica,* Haskins and Blankenship, 1979; Painter *et al.*, 1988; Brown *et al.*, 1989; Ferguson *et al.*, 1989) and to be at least partially under pheromonal control (*A. californica,* Audesirk, 1979).

Ovulation is induced by ELH (*A. californica,* Rothman *et al.*, 1983b), one of approximately nine peptides released during bag-cell activity (*A. californica,* Stuart *et al.*, 1980). Seven of these peptides have been purified from bag-cell extracts or releasates, and chemically characterized. Some of the peptides [*e.g.*, the α-, β-, and γ-bag-cell peptides (BCPs)] have autocrine activities in *in vitro* assays, affecting bag-cell excitability in a temperature-dependent manner (*A. californica,* Rothman *et al.*, 1983a; Redman and Berry, 1991). Others have neurohormonal activities, inducing ovulation (ELH, *A. californica,* Rothman *et al.*, 1983b) and regulating the packaging of eggs into a cordon (δ-BCP, *A. californica,* Nagle *et al.*, 1990). All of the bag-cell peptides that have been examined in an *in vitro* neuroassay system have additional local hormonal activities. These actions appear to be important for transporting the egg cordon through the oviduct (*A. californica,* Alevizos *et al.*, 1991) and for regulating characteristic egg-laying behaviors, although this has not been directly demonstrated *in vivo.*

The bag-cell products that have been characterized to date are encoded by the ELH gene (*A. californica,* Scheller *et al.*, 1983). This gene belongs to a small family of structurally related genes, each of which contains a sequence homologous to ELH and one or more sequences homologous to α-BCP. Members of the ELH family are expressed in a tissue-specific manner in the animal (*A. californica,* Scheller *et al.*, 1983; Mahon *et al.*, 1985), with the ELH gene being expressed in the bag cells and in small populations of neurons in the cerebral and pleural ganglia. Electrophysiological evidence suggests that the cerebral

and pleural cells provide excitatory input onto the bag cells (*A. californica*, Brown *et al.*, 1989; Ferguson *et al.*, 1989); they may, therefore, play a role in regulating egg-laying activity.

Other members of the ELH gene family, the A and B genes, are expressed in the atrial gland (*A. californica*, Scheller *et al.*, 1983; Mahon *et al.*, 1985). Although peptide products of the atrial gland induce egg laying when injected into sexually mature and receptive animals (*A. californica*, Arch *et al.*, 1978), the exocrine nature of atrial gland secretory activity (*A. californica*, Arch *et al.*, 1980; Beard *et al.*, 1982) precludes a hormonal function for these peptides. The present studies and others on pheromonal induction of mating (Painter *et al.*, 1989; also see Susswein and Benny, 1985) suggest that they may serve as sexual pheromones, attracting animals to breeding aggregations and inducing them to mate.

Thus, peptide products of a small family of genes may act to induce and coordinate both male and female reproductive activities: peptide products of the ELH gene, expressed in the neuroendocrine bag cells, act as neurohormones to induce ovulation and, presumably as nonsynaptic neurotransmitters, to regulate the activities and behaviors that accompany egg laying; peptide products of the A gene, expressed in the exocrine atrial gland and secreted onto the egg cordon during deposition, may act as sexual pheromones to attract animals into the aggregation and induce them to mate. Experiments now in progress are aimed at identifying the other attractive atrial gland products and determining whether one or more actually reach the environment to act as sexual pheromones. Dose-response characteristics of the attractants found in eluates will also be examined.

Acknowledgments

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